ß-FRUCTOFURANOSIDASE ACTIVITY IN DISACCHARIDE TRANSPORT MUTANTS OF *STREPTOCOCCUS THERMOPHILUS*

GEORGE A. SOMKUTI* AND DENNIS H. STEINBERG

U.S. Department of Agriculture, ARS, Eastern Regional Research Center, 600 East Mermaid Lane, Philadelphia, PA 19118

SUMMARY. Sucrose transport negative (sucS⁻) mutants were isolated after treating *Streptococcus thermophilus* strain ST128 with *N*-methyl-*N*-nitroso-*N*'-nitroguanidine. The mutants could not grow on sucrose but retained ability to utilize lactose and the capacity to synthesize ß-fructofuranosidase at the constitutive level. The intracellular enzyme required ca. pH 7.0 for optimum activity.

INTRODUCTION

In all dairy fermentations, lactic acid bacteria (lactococci, streptococci, lactobacilli) derive energy primarily from the stepwise bioconversion of lactose to lactic acid. However, in the commercial production of yogurt, another fermentable carbohydrate is sucrose which is frequently added as a sweetener to increase consumer acceptance (McGregor and White, 1986). The fermentation of carbohydrates in yogurt is accomplished by *Streptococcus thermophilus* in mutualistic partnership with *Lactobacillus bulgaricus*.

Various aspects of lactose transport and metabolism and their genetic control in *S. thermophilus* have been studied extensively by several laboratories. These include studies on lactose and galactose transport (Somkuti and Steinberg, 1979a; Hutkins *et al.*, 1985a; Hutkins and Ponne, 1991), characterization of ß-galactosidase (Somkuti and Steinberg, 1979b; Smart and Richardson, 1987), genetic cloning in *Escherichia coli* of the ß-galactosidase (Herman and McKay, 1986; Schroeder *et al.*, 1991) and lactose transport genes (Poolman *et al.*, 1989), and studies on the enzymes of galactose metabolism (Thomas and Crow, 1984; Hutkins *et al.*, 1985b), including the recent cloning of genes for mutarotase and UDPglucose-4-epimerase (Poolman *et al.*, 1990). Because of its food grade status, *S. thermophilus* has been recommended as a source of thermostable ß-galactosidase (Greenberg and Mahoney, 1982; Chang and Mahoney, 1989; Somkuti and

Steinberg, 1990) for the alleviation of lactose malabsorption, a condition that affects a large segment of the world's adult population (Hourigan, 1984).

In comparison, information on sucrose utilization and metabolism in *S. thermophilus* is much more limited (Thomas and Crow, 1983; Hutkins and Morris, 1987; Amoroso *et al.*, 1988) and there have been no reports on the properties of ß-fructofuranosidase of this microorganism. In the present paper, we describe the isolation of disaccharide transport defective mutants of *S. thermophilus* and the hydrolysis of sucrose by permeabilized cells.

MATERIALS AND METHODS

Culture and growth conditions

The culture of *S. thermophilus* ST128 used as the source of ß-fructofuranosidase (ß-D-fructofuranoside fructohydrolase, EC 3.2.1.26) and ß-galactosidase (ß-D-galactoside galactohydrolase EC 3.2.1.23) was from our laboratory collection. Phenotypically, this strain is characterized by fermentation of sucrose (Suc^+), lactose (Lac^+) and glucose (Glu^+), and inability to ferment galactose (Gal^-) or fructose (Fru^-). The culture was maintained in basal medium (BM) containing 3% tryptone (Difco), 1% yeast extract (Difco), 0.2% beef extract (Difco), and 0.5%KH_2PO_4 and supplemented with 0.5% sucrose (BMS) or lactose (BML) as needed. Medium pH was adjusted to 6.5 before sterilization. Incubation was at 37°C in an atmosphere of 5% CO_2 for 24 h. Between transfers, cultures were refrigerated.

Mutagenesis and screening for mutants

Bacteria from 10 ml of exponential culture (OD_{660}= 0.4) grown in BMS were suspended in 5 ml 50mM potassium phosphate buffer (PO, pH 7.0). NNG (*N*-methyl-*N*-nitroso-*N*'-nitroguanidine) was added to a final concentration of 0.5 mg/ml, which killed about 85% of the bacteria. After 60 min at 37°C, cells were harvested, washed twice with PO buffer and resuspended in 10 ml of peptone (Difco, 1 mg/ml) water. Serial dilutions were done and samples were plated on BML plates with 1.5% agar to count survivors. After 2 days at 37°C ca. 500 clones picked randomly were transferred into 96-well BMS and BML microtiter plates (300 µl/well) to check for loss of sucrose fermentation ability (Suc^-). Clones showing Suc^- phenotype (inability to grow in BMS) were classified as putative sucrose transport ($sucS^-$)defective mutants. Similar experiments were done to select lactose transport defective ($lacS^-$) mutants that lost the ability to grow in BML (Lac^-) but grew normally in BMS.

Permeabilization of whole cells

In order to assay enzyme activities *in situ*, bacterial cells were permeabilized as described previously (Somkuti and Steinberg, 1979b). After exposure to the acetone-toluene (9:1 v/v) mixture, cells were pelleted and washed twice with 5 ml PO buffer before resuspending in 1 ml of the same. Cell suspensions were prepared on the day of enzyme assays and kept on crushed ice until use.

Enzyme assays

The ß-fructofuranosidase (ß-fru) and ß-galactosidase (ß-gal) activities of putative $sucS^-$ and $lacS^-$ mutants were measured by incubating suspensions of permeabilized cells in 5% sucrose or 5% lactose in PO buffer containing 1mM $MgCl_2$ (POM), at 50°C for 10 min. Reactions were terminated by pelleting cells in an Eppendorf microfuge and removing the supernatants. Activity was determined by measuring the amount of glucose released under experimental conditions with a Glucose HK Kit (Sigma Diagnostics) according to the manufacturer's instructions. A unit of ß-fru or ß-gal activity was defined as the amount of enzyme that released 1 µmol glucose per min from its respective substrate. Protein was measured by the method of Lowry (Lowry *et al.*, 1951) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

To simplify experimental design, the optimum pH range for ß-fru activity was first established. Permeabilized cells of *S. thermophilus* ST128 wild type ($sucS^+$. $lacS^+$) were tested for the ability to hydrolyze sucrose between pH 5.0 and 8.5 (Fig. 1). The optimum range for ß-fru activity was pH 6.5-7.5. Since this was similar to the optimum pH range (pH 6.5-8) reported previously for the ß-gal of this microorganism (Somkuti and Steinberg, 1979b; Ramana Rao and Dutta, 1981; Greenberg and Mahoney, 1982), both ß-fru and ß-gal activities were determined in POM buffer at pH 7.0.

The selection of disaccharide transport mutants ($sucS^-$ and $lacS^-$) was based on loss of ability to grow in BMS or BML. The NNG treatment of $sucS^+$.$lacS^+$ ST128 cells yielded two $sucS^-$ mutants and three $lacS^-$ mutants. In $sucS^-$ mutants lactose transport was apparently unimpaired as these cultures continued to ferment lactose. By comparison, $lacS^-$ mutants retained ability to grow in BMS, indicating the presence of an intact sucrose transport system. Both $sucS^-$ and $lacS^-$ mutants apparently lost the ability to grow in a glucose medium. However, the capacity to ferment glucose apparently remained unimpaired in both types of mutants following the

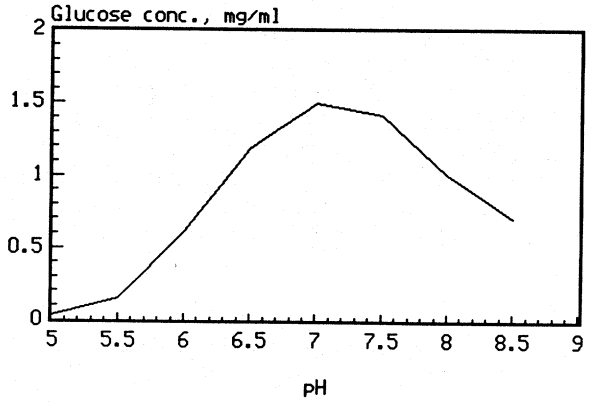

Fig.1. Effect of pH on sucrose hydrolysis by *S. thermophilus* ST128. Identical amounts of permeabilized cells were incubated with 1% sucrose in POM for 2 h at 50°C. Glucose was measured in cell-free supernatants.

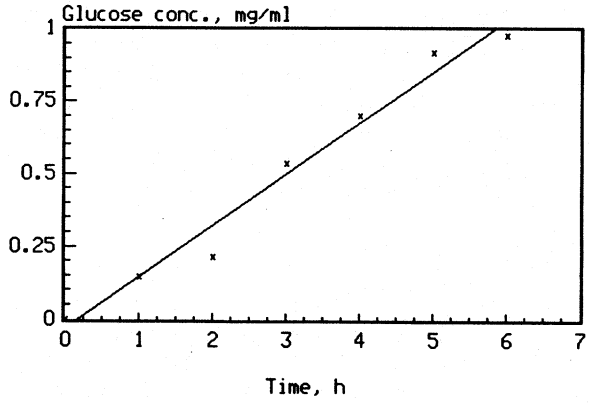

Fig.2. Time course of sucrose hydrolysis by permeabilized cells of ST1281 lacS$^-$ mutant strain (1% sucrose in POM, pH 7.0, 37°C).

Table 1. ß-fructofuranosidase and ß-galactosidase levels in disaccharide transport mutants of *S. thermophilus*

Culture	Phenotype	Growth medium	Specific activity* ß-fru	ß-gal
ST128	sucS$^+$.lacS$^+$	BML	0.53	28.6
		BMS	1.58	16.5
ST1281	sucS$^-$.lacS$^+$	BML	0.65	29.0
ST1282	sucS$^+$.lacS$^-$	BMS	1.48	15.6

* Specific activity was defined as μM of glucose released per mg of protein per min. Experimental conditions were as described in *Materials and Methods*

transport of either disaccharide into the cell interior.

The rate of sucrose hydrolysis by a $sucS^-$ mutant was checked with permeabilized cells of ST1281 (Fig. 2). Under the experimental conditions used, ca. 10 per cent of the substrate was hydrolyzed after 6 h of incubation.

The ß-fru and ß-gal activities of the wild type ST128 ($sucS^+.lacS^+$) culture as well as the mutant cultures ST1281 ($sucS^-.lacS^+$) and ST1282 ($sucS^+.lacS^-$) were determined *in situ* using permeabilized cells prepared from exponentially growing populations. The data in Table 1 show that both ß-fru and ß-gal enzymes were present in permeabilized cells regardless of individual phenotype. This confirmed that the loss of ability to ferment a disaccharide resulted from damage to its specific disaccharide transport system and not from the lack of disaccharide hydrolyzing enzyme or loss of glucose catabolism.

The results also provided information on the inducible nature of ß-fru in *S. thermophilus*. The specific ß-fru activity of ST128 ($sucS^+.lacS^+$) was ca. 3 times higher in BMS grown cells (fully induced) than in BML cells that produced ß-fru apparently at the constitutive level. Furthermore, the same constitutive level of ß-fru was present in ST1281 ($sucS^-.lacS^+$) which failed to grow in BMS. The corresponding values obtained with ß-gal confirmed the inducibility of this enzyme which was reported previously for various strains of *S. thermophilus* (Somkuti and Steinberg, 1979b; Tinson *et al.*, 1982; Smart *et al.* 1985). Overall, the constitutive and induced specific activity levels of ß-gal were significantly higher than those determined for ß-fru in *S. thermophilus* and its mutants. Neither ß-fru or ß-gal activity was detectable in cell-free culture filtrates confirming the intracellular nature of the two enzymes.

Since *S. thermophilus* is a food grade bacterium and consumed as a natural component of dairy foods such as yogurt and a variety of cheeses, it may be viewed as a microcarrier of enzymes that could be added directly to food products without the need for enzyme purification. Indeed, permeabilized cells of *S. thermophilus* $lacS^-$ mutants may represent an ideal source of ß-gal for reducing the lactose content of milk (Somkuti and Steinberg, 1990). Because of the higher sweetening power of fructose over sucrose (Grenby, 1983), the $sucS^-$ mutants *S. thermophilus* may likewise find uses as food grade sources of ß-fru, well suited for *in situ* hydrolysis of sucrose in food products or the preparation of high fructose sweeteners. Such applications will require detailed characterization of the molecular properties of ß-fru and the development of *S. thermophilus* strains with significantly higher constitutive levels of ß-fru activity.

REFERENCES

Amoroso, M.J., Manca de Nadra, M.C. and Oliver, G. (198-8). Milchwissenshaft 43, 626-631.

Chang, B.S. and Mahoney, R.R. (1989). J. Dairy Res. 56, 117-127.

Greenberg, N.A. and Mahoney, R.R. (1982). J. Food Sci. 47, 1824-1828.

Grenby, T.H. (1983). In "Developments in Sweeteners - 2"; T.H. Grenby, K.J. Parker and M.G. Lindley, Eds.; Applied Science Publishers: London and New York, p59.

Herman, R.E. and McKay, L.L. (1986). J. Appl. Environ. Microbiol. 52, 45-50.

Hutkins, R., Morris, H.A. and McKay, L.L. (1985a). Appl. Environ. Microbiol. 50, 772-776.

Hourigan, J.A. (1984). Aust. J. Dairy Technol. 39, 114-120.

Hutkins, R., Morris, H.A. and McKay, L.L. (1985b). Appl. Environ. Microbiol. 50, 777-780.

Hutkins, R.W., Morris, H.A. (1987). J. Food Prot. 50, 876-884.

Hutkins, R.W. and Ponne, C. (1991). Appl. Environ. Microbiol. 57, 941-944.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). J. Biol. Chem. 193, 265-275.

McGregor, J.V. and White, C.H. (1986). J. Dairy Sci. 69, 698-703.

Poolman, B., Royer, T.J., Mainzer, S.E. and Schmidt, B.F. (1989) J. Bacteriol. 171, 244-253.

Poolman, B., Royer, T.J., Mainzer, S.E. and Schmidt, B.F. (1990) J. Bacteriol. 172, 4037-4047.

Ramana Rao, M.V. and Dutta, S.M. (1981). J. Food Sci. 46, 1419-1423.

Schroeder, C.J., Robert, C. Lenzen, G., McKay, L.L. and Mercenier, A. (1991). J. Gen. Microbiol. 137, 369-380.

Smart, J.B., Crow, V.L. and Thomas, T.D. (1985). NZ J. Dairy Sci. Technol. 20, 43-56.

Smart, J. and Richardson, B. (1987). Appl. Microbiol. Biotechnol. 26, 177-185.

Somkuti, G.A. and Steinberg, D.H. (1979a) J. Food Prot. 42, 881-887.

Somkuti, G.A. and Steinberg, D.H. (1979b) J. Appl. Biochem. 1, 357-368.

Somkuti, G.A. and Steinberg, D.H. (1990) Biotechnol. Appl. Biochem. 12, 351-356.

Thomas, T.D. and Crow, V.L. (1983). FEMS Microbiol. Lett. 17, 13- 17.

Thomas, T.D. and Crow, V.L. (1984). Appl. Environ. Microbiol. 50, 777-780.

Tinson, W., Hillier, A.J. and Jago, G.R. (1982). Aust. J. Dairy Technol. 37, 8-13.